Abstract

The growing wealth of information regarding the influence that physicochemical characteristics play on nanoparticle biocompatibility and safety is allowing improved design and rationale for their development and pre-clinical assessment. Accurate and appropriate measurement of these characteristics accompanied by informed toxicological assessment is a necessity for the development of safe and effective nanomedicines. While particle type, formulation, and mode of administration dictate the individual causes for concern through development, the benefits of nanoformulation for treatment of the diseased state are great. Here we have proposed certain considerations and suggestions which could lead to better informed pre-clinical assessment of nanomaterials for nanomedicine, as well as how this information can and should be extrapolated to the physiological state of the end user.

Key Words

Nanoparticles, Nanotoxicology, Nanomedicine

Introduction

The application of nanotechnology in a healthcare setting offers many novel therapeutic strategies that may improve existing therapies and diagnostics. Desirable physicochemical characteristics (PCC) of nanoparticles that can translate to medical benefits include structural and stability related properties to improve bioavailability, biodistribution and reduce clearance [1, 2]. Additionally, there are opportunities for targeted therapies, which may reduce undesirable effects in other cell types, and co-formulation that may alleviate pill burden in diseases such as HIV as well as simplifying dosing strategies by enabling parenteral long-acting depot formulations.

While there are obvious advantages to the application of nanotechnology, it is entirely possible that it will not be a case of “one size fits all” and that certain drugs may only be compatible with particular nanoparticles or nanoformulation strategies. Indeed, nanomedicine has attracted recent interest in the fields of precision- and personalised-medicine [3].

Size, charge, hydrophobicity and shape are some of the numerous characteristics that can be tuned by the manufacturing process. Modification of these properties can alter the biological interactions of these nanoparticles. For example, uptake of gold nanoparticles by epithelial cells has been shown to be size-dependent where the rate increases with decreasing nanoparticle size [4], and hydrophobic modification of glycol chitosan nanoparticles increased uptake in cancer cells [5].

The heterogeneity of nanoparticles being produced by various inventors is a major advantage as it provides many options for the treatment of a broad range of diseases by enabling many strategies for the formulation of therapeutic compounds as well as allowing interactions with many therapeutics. However, the broad spectrum of nanoparticle classes, in addition to their physicochemical characteristics, presents a challenge in determining their biocompatibility. A balance should be found between nanoparticle characteristics that favour the delivery of therapeutic agents while simultaneously not resulting in issues around either toxicity or undesirable interactions with the immune system. Clearly therefore, a rational understanding of how

nanoparticle physical properties relate to their biological interactions is required for the efficient development of beneficial materials.

Interaction of nanoparticles with components of the immune system

There are many well-described interactions of nanoparticles with cells of the immune system [6]. The reasons for these interactions may be linked to specific nanoparticle properties, in particular size and charge [7-9]. Many nanoparticles are within the size range of microorganisms that the immune system has evolved to recognise, with many signatures in common with invading pathogens [10].

The mechanism by which nanoparticles are internalised varies between immune cell types. As demonstrated in **Figure 1** this includes, but is not limited to, phagocytosis, endocytosis, passive uptake, and receptor-interaction based uptake. Phagocytosis (a process performed by macrophages, monocytes, neutrophils, dendritic cells, and mast cells) leads to the capture and internalisation of nanoparticles in phagosomes which in turn undergo lysosomal degradation [11]. While this is an effective tool for removing biological pathogens, nanoparticles are not so simply degraded. The pH environment of the phagolysosome may affect the stability of the nanoparticle leading to the release of metallic ions in the case of metallic nanoparticles [12]. These in turn can disrupt mitochondrial processes and generate reactive oxygen species through Fenton type reactions [12]. A similar effect can be observed in clathrin-mediated [13] and clathrin-independent endocytosis [14] where degradation occurs following lysosomal fusion with the endosome. Caveolin-mediated endosomes bypass lysosomal degradation [15] the mechanism of which is being explored for its potential for intracellular delivery of nanomaterials [16].

Nanoparticles which passively enter the cell, or those which escape phagocytic/endocytic vesicles are then able to come in direct contact with intracellular proteins and organelles [17], with the potential to interact in a detrimental manner. Internalised nanoparticles have been shown to interfere with the normal autophagic process [18] and also as a result modulate the NLRP3 inflammasome [19].

Interaction with certain classes of cell surface receptors leads to the internalisation of nanoparticles, usually displaying certain surface motifs [20] although this is not a necessity as scavenger receptors have been shown to bind polystyrene via the action of macrophage receptor with collagenous structure (MARCO) [21]. Activation of receptor associated pathways as a result of the binding of nanoparticles has been demonstrated where TLR4 signal transduction following the binding of polyethylenimine-coated SPIONs [22].

In addition to size and charge, hydrophobicity has also been demonstrated to be an important factor in the recognition of nanoparticles by the immune system [23]. As many intracellular danger-associated molecular patterns (DAMPs) are hydrophobic in nature their release upon cellular damage signals to the immune system to respond to this damage. Hydrophobic nanoparticles have been shown to more likely induce an immune response than those which are less hydrophobic [24]. As more classes/types of nanomaterials are created it is entirely possible that additional nanoparticle characteristics will be recognised for their association with biocompatibility, and

nanoparticles may be stratified for their interactions with the immune system by class-specific properties.

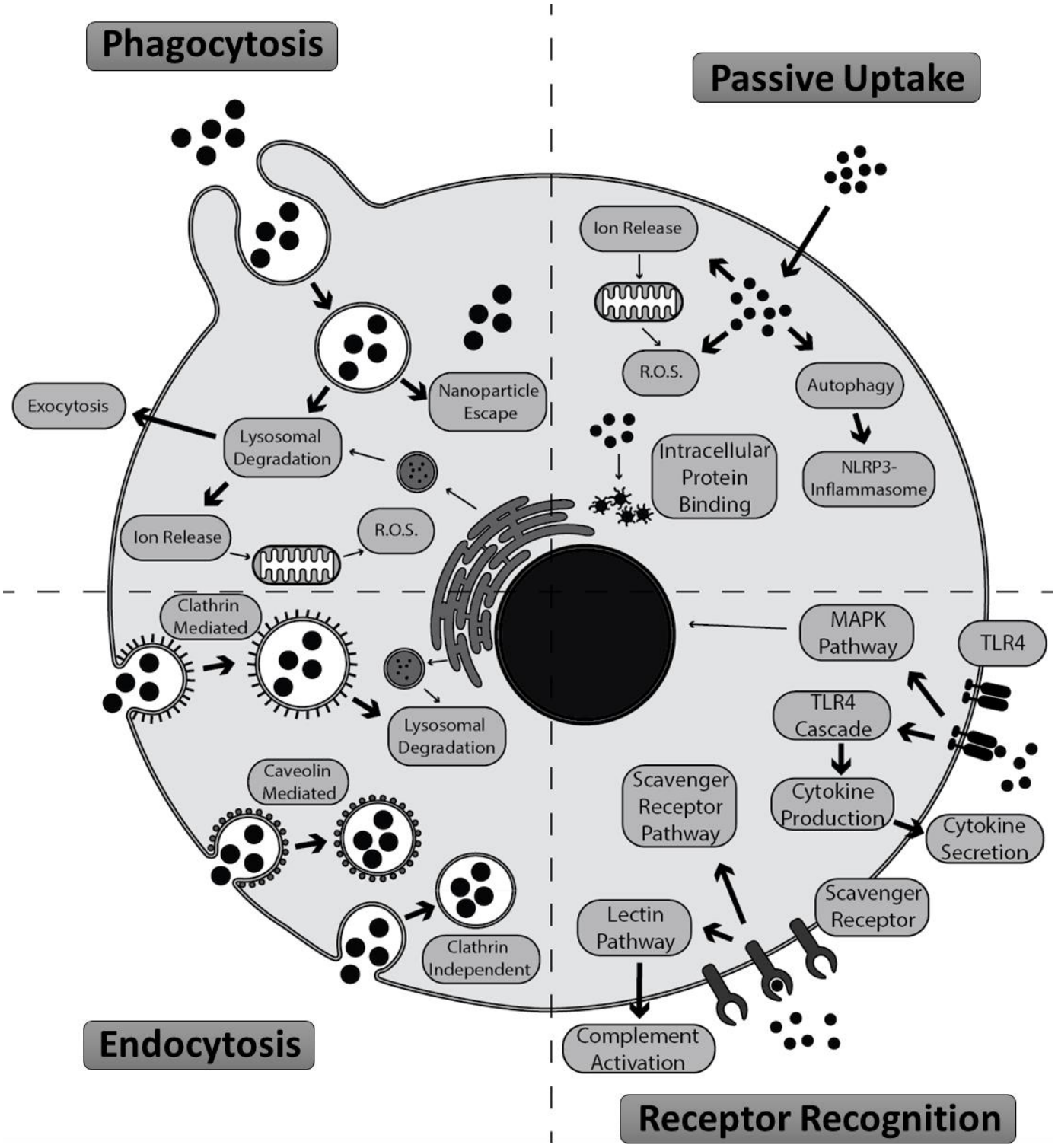


Figure 1 – Routes of entry determine nanoparticle intracellular effects, and extracellular consequences. *Internalisation of nanomaterials includes, but is not limited to, endocytosis (including phagocytosis), receptor-binding, and passive uptake. The fate, and associated intracellular effects of these mechanisms include lysosomal degradation, generation of by-products such as metal ions which can induce reactive oxygen species generation in mitochondria, direct interference with*

intracellular processes involved in autophagy and the NLRP3-inflammasome, and activation of intracellular cascades such as the scavenger receptor pathway, TLR4 cascade, MAPK pathway, and the lectin pathway. Extracellular consequences include exocytosis, cytokine secretion, and complement activation. Effects displayed here are non-exhaustive, some being ubiquitous and not limited to individual modes of entry to the cell. Acronyms used; R.O.S. - reactive oxygen species, NLRP3 - NLR family pyrin domain containing 3, TLR4 - Toll-like receptor 4, MAPK - mitogen activated protein kinase.

Stimulation of the immune system by nanoparticles

As foreign substances to the body, nanoparticles may be recognised by the immune system and removed with the possibility to stimulate immune responses by both innate and adaptive mechanisms. Immunogenicity of nanomaterials is largely reliant on their route of administration, as this greatly affects their presentation to the immune system [25].

Intravenously administered nanomedicines come directly into contact with plasma proteins which, depending on; particle characteristics, composition and the method of preparation result in protein binding to the nanomaterial surface [26, 27]. While the formation of a "protein corona" is ubiquitous to all nanomaterials when subjected to a biological medium, it has been shown to have important implications for many aspects of nanoparticle-biological interactions *in vivo* [28] such as activating complement [29], and differential cellular uptake dependent on coronal composition [30]. Recent work by Tenzer *et al.* [31] has furthered the understanding of the temporal composition of the nanoparticle corona. While this work was unable to also investigate the "soft corona", the presence of which further increases the complexity of nanoparticle presentation to the immune system, it has shown that the coronal structure changes as a function of time affecting the material's pathophysiology.

Currently, nanoparticle antigenicity is not well understood. The process of antigenicity involves plasma B cells to generate antibodies against the nanoparticle, or functional groups, such as peptides, attached to the particle surface [32]. Since nanoparticle specific antibodies should only influence the effectiveness of particle-based products, for example by modulating cellular interactions or biodistribution, it is more probable that antibodies that recognise the functional ligands present on the nanoparticle surface may cause similar clinical results as those seen for biotechnology-derived therapeutics [33, 34]. Anti-nanoparticle immunoglobulin formation has been reported. Polyclonal C_{60}-specific antibodies with a subpopulation cross-reacting with the C_{70} fullerene have been demonstrated, as well as monoclonal antibody responses to C_{60} fullerenes [35, 36]. PEGylation (the functionalization of nanoparticles with polyethylene glycol chains) has been used to reduce their immunogenic potential, but the production of anti-PEG antibodies has also been reported [37, 38].

Examples of specific nanoparticle properties influencing immune stimulation have been reported. For instance, cationic nanoparticles have a greater potential to induce inflammatory responses than neutral or anionic nanomaterials. An example of this are positively charged 4.5 polyaminoamine (PAMAM) dendrimers do not cause the secretion of cytokines by human leukocytes [39] whereas negatively charged liposomes cause the production of interleukin-2 and interferon gamma [40]. CD4

expressing T lymphocytes, known as T helper cells (Th), are a key cell type for the secretion of cytokines. Th cells may be divided into TH1 and TH2, which produce Th1-type or Th2-type cytokines, respectively. Several studies have addressed the influence of nanoparticles on Th1 and Th2 responses [41-43]. Th1 cells activate and support cell-mediated immunity, killing virally infected or malignant cells while Th2 cells induce humoral immunity and support antibody production by B cells. Large (>1 μm) industrialized particles induce Th1 responses, whereas smaller (<500 nm) particles are linked with Th2 response [44]. In contrast, engineered nanomaterials including 80 nm and 100 nm nanoemulsions [45, 46], 123 nm self-assembled dendrimers [47], 270 nm poly(lactic-co-glycolic) acid (PLGA) [48], and 500 nm PLGA [49] induce Th1 response. Other engineered particles (e.g. 5 nm generation-5 PAMAM dendrimers) do not demonstrate *in vivo* inflammatory reactions, but enhance immunoglobulin production and weakly induce Th2 cytokine production [50]. The potential contradiction in these findings warrants further investigation to establish whether this is due to nanoparticle characteristics or varying experimental approaches.

Macrophages are able to phagocytose nanoparticles, the size of which influences the observed stimulatory effects most likely due to size dependent thresholds on the phagocytic capacity of macrophages [51]. Nanoparticles of the range 200-600nm induce IFNγ, favouring a Th1 type response while 2-8μm particles induce IL-4 secretion and favour a Th2 type response [52]. From an immunological context, this may be linked to the differential uptake of these nanomaterials as smaller nanoparticles may differentially accumulate in macrophages compared to larger nanoparticles [51, 53].

Unwanted immune stimulation is a hurdle for the development of some nanomaterials, but it does also present an opportunity for the formulation of certain therapeutics, in particular, antigens to be utilised in vaccines. The use of nanoparticles as adjuvants has been reported by numerous studies. Poly(methyl methacrylate) (PMMA) nanoparticles have been shown to induce long-lasting antibody titres in HIV-2 whole virus vaccine in mice, and the antibody response was 100-fold higher than that of standard adjuvant [54]. Similarly, the levels of specific antibodies produced in the immunisation of animals with colloidal gold conjugated antigens were higher than that generated by classical adjuvants while the amount of antigen required to achieve this response was an order of magnitude lower than for immunisation with a standard adjuvant [55]. The reasons for this may be due to greater accumulation of the antigen in cells such as dendritic cells allowing greater presentation of the therapeutic antigen to the immune system.

Concerning the formulation of vaccines, the generation of inflammation is desirable when nanoparticles are targeted to dendritic cells (DCs). DCs have the ability to induce and modulate the immune response. DCs play a key role in the activation of T cells and as such are a principal target for most vaccines. Utilization of “danger signals” in vaccine design (DC activating non-host signals) combined with specific antigen to induce the desired immune response type is a common approach [56]. As mentioned earlier, nanoparticle size can govern their immunostimulatory profile with plasmacytoid DCs (pDCs) showing preferential uptake of nanoparticles <200nm, resulting in the production of IFNα while phagocytosis by monocytic DCs (mDCs) of 500-1000nm particles induced TNFα [57]. Similarly, Gadolinium containing nanoparticles have been reported to possess antitumour activity resulting from their ability to induce the maturation of immature DCs [39]. Stimulation of DCs by TMC-TPP nanoparticles has been shown to induce differentiation of T cells to inflammatory TH17 [58]. As an alternative proinflammatory pathway to TH1- and TH2-type responses the IL-17

mediated cascade offers a further mechanism for enhanced effect as an adjuvant. The opposite effect was observed following DC stimulation by PLGA nanoparticles where not only was TH17 differentiation inhibited but also differentiation of naïve $CD4^+$ T cells to $FoxP3^+$ T cells (Treg cells) was observed. The anti-inflammatory role which Treg cells play in self-antigen tolerance, inhibition of T cell response, cytokine release, as well as NK and $CD4^+$ cell activity would not be favourable for a vaccine-based application. Determination of the favourable characteristics of nanoparticles that are correlated with the desired effect is vital to the development of future nanomaterials.

The application of knowledge regarding the biodistribution and accumulation of nanomaterials in vivo [59] is highly important when interpreting immunogenicity not only regarding use as adjuvants but for general safety. Passive and active accumulation of nanoparticles in multiple sites increase the concern of off-target toxicity. The relationship between administration route and biodistribution of nanoparticles is intrinsically linked, and to date, there exists no thorough evaluation of route of administration, and how it relates to cytotoxicity following tissue accumulation.

Suppression of the immune system by nanoparticles

Immunosuppression can be the result of numerous biological effects both directly and indirectly resulting from the systemic presence of nanomaterials. Identification of immunosuppressive effects of nanoparticles is complicated by the fact that these effects may be subtle and not identified until long-term exposure to nanoparticles. Thorough, long-term study is required for the evaluation of immune suppression and careful consideration of the factors involved is required. Unintended immune suppression is an undesirable outcome in areas where patients may already be immunocompromised such as in cancer and HIV infection. Identification of undesirable immunosuppressive properties of engineered nanomaterials may be an important component of their preclinical evaluation. The current knowledge of immunosuppression by nanoparticles has been recently reviewed [60, 61] but some key examples are elaborated in this section.

The possible mechanisms by which immunosuppression may occur can be linked to direct anti-inflammatory activity of nanoparticles (silver nanoparticles [62]), nanoparticles with antioxidant activity (cerium oxide nanocrystals [63]), those with anti-cytokine activity (citrate-stabilized gold nanoparticles [64, 65]), inhibitors of cell-mediated immunity (iron oxide nanoparticles [66]), those that interfere with normal antigen response (multi-walled carbon nanotubes [67]), inducers of myelosuppression (doxorubicin bound to polyisobutyl [68]), and those cytotoxic to immune cells (zinc oxide [69]). The range of nanomaterials associated with these outcomes is quite broad, some of which mediate their effects via multiple mechanisms [60].

The generation of oxidative stress following accumulation in cells is the primary mode of toxicity for some nanomaterials as demonstrated in **Figure 1**. Generation of reactive oxygen species is linked with activation of the NLRP3 inflammasome [70] which in turn triggers release of proinflammatory cytokines IL-1β and IL-18 [71], leading to immune stimulation. Certain nanoparticles, including cerium oxide and gold nanoparticles [72, 73], have been found to have antioxidant activity due to their ability to quench free radicals.

Nanoparticles such as citrate-stabilised gold have demonstrated anti-cytokine activity by sequestering extracellular IL-1β [65] thereby inhibiting responses initiated by IL-1β in certain cell lines. Additionally, interference with TLR9 translocation, via binding of the signalling regulator high-

mobility group box-1 (HMGB1) [64], therefore diminishing the effect of TNFα generated by an immune stimulant (CpG-ODN). The binding potential of gold nanoparticles is a commonality that underpins the proposed mechanisms.

Fullerenes [74] and carbon nanotubes [67] have been strongly associated with immunosuppression by interfering with the normal response of immune cells to antigens while many dendrimers are being studied to exploit their immunosuppressive qualities [75]. Large amine-and hydroxyl-terminated dendrimers were shown to be able to inhibit inflammation via inhibition of cyclooxygenase (COX1 and COX2) in a concentration-dependent manner [76].

API involvement in nano-immunomodulation

While inadvertent immunosuppression could result in catastrophic consequences, especially in diseased states with associated immunocompromisation, it may be desirable when utilized in the treatment of inflammatory disorders and autoimmune disease. The clinical potential to improve transplant acceptance by the prevention of allergic responses would be invaluable, and current progress shows great promise by utilizing nanocarriers for the delivery of immunomodulating agents such as rapamycin [77] or donor antigens for the induction of transplant tolerance utilizing vaccine/adjuvant principals [77, 78].

Controlled delivery of active pharmaceutical ingredients (APIs) to target sites using nanocarriers is an ongoing challenge. Underpinning this is the need to assess potential and effects of the accumulation of APIs in off-target tissues or immune cells. Polymeric and liposomal carriers are well known to have a higher accumulation in the lymphatic system [79] wherein their potential to interact with lymphocytes in a non-beneficial manner poses cause for concern. Lopinavir, a protease inhibitor used in the treatment of HIV the nanoformulation of which is currently in development [80] has been shown to induce cytokine secretion from various immune cells [81], and Rapamune [82] a nanoformulation of rapamycin used as an immunosuppressant, although possessing antipodal immunological effects are both pertinent examples of APIs whose impacts need to be assessed separately to their carrier system. Following accumulation or degradation of either API or carrier, any associated immunomodulatory effects could become apparent. Immunostimulatory or immunosuppressive properties of the API potentially enhance, or mask those of the carrier system and vice versa. Whether they are by design or unintentional, such effects need to be fully accounted for.

Interaction of nanoparticles with components of the blood

Many nanoparticles have been shown to influence a number of haematological components and processes [83]. In their normal homeostatic role platelets facilitate coagulation and are involved in the thrombogenic process to stop bleeding [84]. Platelet activation and thrombus formation have been found to occur in response to nanomaterials in the systemic circulation [85]. Platelet aggregation following the activation of glycoprotein integrin receptor GPIIb/IIIa has been observed for both single walled carbon nanotubes (SWCT) and multi-walled carbon nanotubes (MWCNT) in a particle size-dependant manner [85]. Platelet activation has also been strongly associated with GPIIb/IIIa activation by silver ions released from silver nanoparticles [86, 87] and increased

intracellular calcium ion concentration resulting from silica nanoparticles [88]. The interaction of charged polystyrene latex nanoparticles has been found to cause physical bridging of platelets in a GPIIb/IIIa independent manner [89].

The properties of size, charge, hydrophobicity, and the presence of certain surface groups can determine thrombogenicity of nanoparticles resulting from altering prothrombin times and activated partial thromboplastin times, as well as the mechanism by which coagulation is induced [83]. Anionic polystyrene latex nanoparticles caused platelet aggregation via upregulation of adhesion receptors while their cationic counterparts initiated platelet aggregation following destabilization of cell membrane integrity [90]. Amine-functionalized nanoparticles reduced thrombin production via depletion of factors VII and IX in a size dependent manner [91]. It has been shown that these characteristics hold greater influence over thrombogenicity than does the basic composition of a given material [83]. Cationic, but not neutral or anionic, PAMAM dendrimers cause platelet aggregation [92, 93]. The size-dependence of polystyrene nanoparticles to cause coagulation has been suggested because 220nm but not 24nm particles exhibited this effect [91].

Links between immunological and haematological systems

Immunological and haematological systems do not function in isolation and have evolved to work cooperatively to both detect infection and ensure resolution of the response. There are a number of examples of how nanoparticles interact with one system, which in turn activates the other.

Leukocyte procoagulant activity

Leukocytes play key roles in the regulation of thrombin formation [94] having an influence over inflammation, wound healing and atherosclerosis. Monocytes and neutrophils [95] are recruited by activated platelets at sites of thrombogenesis. This is achieved via recognition of P-selectin on the activated platelet by leukocyte P-selectin glycoprotein ligand (PSGL)-1 resulting in conformation changes in β2 integrins [96] leading to potent procoagulant activity. Induction of tissue factor synthesis, the presence of which is necessary for the production of thrombin, leads to thrombus formation [97].

Contamination of materials can have a great effect on the pro-coagulant activity of leukocytes. It has been shown that the presence of endotoxin confers leukocytes with considerable procoagulant activity [98]. Contamination of nanomaterials by endotoxin may cause false positives in many immunological assays and it has been demonstrated that cationic PAMAM dendrimers have been shown to enhance the procoagulant activity induced by endotoxin [99, 100].

Complement activation

The complement system is a vital component of the innate immune system with functions involved in homeostasis, pathogen recognition, and determining the appropriate immune response be it innate or adaptive [101]. Nanoparticles have been shown to activate the complement system following intravenous injection [102]. It is a multicomponent system made up of over 30 membrane-associated and soluble proteins [103]. Complement activation leads to sequential reactions resulting in the formation of C3a and C5a anaphylatoxins which exert multiple inflammatory responses which

include the recruitment of phagocytes [103]. Numerous studies have pointed towards complement activation being a contributing factor in the development of hypersensitivity and anaphylaxis as a response to the systemic presence of nanoparticles [6, 104, 105]. Hypersensitivity reactions have been reported for the liposomal formulation Doxil™ [106] there is evidence that this is mediated by complement activation [105]. It has been described that polymeric nanoparticles consisting of PEG-PL (block copolymers of poloxamer and poloxamine) can activate complement exclusively via the lectin pathway [107]. This mechanism is normally reserved for the recognition of repeating and charged motifs of certain polysaccharides [108].

Platelet activation and immune stimulation

The link between platelet activation and immune stimulation is multifactorial and double-edged. While thrombogenesis can influence immune stimulation, along with various thrombogenic factors being able to inhibit or augment immune responses, the opposite is also true where immune stimulation increases thrombogenic potential. Proinflammatory cytokines and endotoxin induce tissue factor production on leukocytes which in turn initiates extrinsic coagulation via thrombin (FIIa) generation [109]. Complement activation leads to enrichment of plasma membrane surfaces with negatively charged phospholipids which have been shown to amplify coagulation [110].

Thrombogenic function is just one of the numerous activities which platelets can play within homeostasis. The involvement of platelets within immune stimulation has gained recognition in recent years [111, 112]. Platelets carry numerous receptors including TLRs and express immunomodulatory molecules and cytokines [113]. An example of how nanoparticles may cause immune stimulation via platelets has been demonstrated previously with multi-walled nanotubes (MWNT). MWNT were shown to induce the release of platelet membrane microparticles capable of stimulating other immune cells [114]. Further studies are warranted on the interaction of platelets and immune cells with respect to nanoparticle effects on both cell types.

Haemolytic potential

The mechanisms of nanoparticle-mediated haemolysis are not fully understood. Haemolysis is the result of damage to red blood cells and may be used as a measure of cell viability in response to contact with materials in addition to possibly leading to anaemia [115]. Many studies currently exist which describe the haemolytic potential of various nanomaterials but only some suggestions exist concerning their mode of action [104] primarily membrane disruption via interactions with red blood cell membrane phosphatidylcholine [116, 117]. Charge has been shown to strongly influence whether nanoparticles cause haemolysis. This process has been related to the disruption of cell membranes via pore formation following the integration of charged nanoparticles into existing membrane defects [118]. The potential for nanoparticles to become ionised [119], surface groups [116, 117], and cationic charge seem to be parameters likely to have an effect. Materials which exhibit this trend include silica nanoparticles [120, 121] as well as numerous others via the presence of unprotected amines on the nanoparticle surface such as PAMAM [122], carbosilane [123], polypropylene imine [124], and polylysine [125] dendrimers, which have been associated with erythrocyte damage in a dose dependent manner. The haemolytic potential of silver nanoparticles has been well described in numerous sources [86, 119, 126]. It has been demonstrated that with increasing hydrophilicity the haemolytic potential increases [127]. The presence of a protein corona has been shown to have a protective effect, and the haemolytic potential of gold nanoparticles

featuring both hydrophobic and hydrophilic surface functionalization was reduced [127]. This effect has also been described by Tenzer *et al.* wherein the presence of protein corona on silica nanoparticles negated their haemolytic activity as well as a reduced level of thrombocyte activation compared to pristine nanoparticles [31].

Challenges in assessing the biocompatibility of novel, engineered, nanoparticles

Contamination

The potential for nanomaterial contamination is intrinsically linked to the associated manufacturing process. Bacterial endotoxin is a contaminant which elicits a strong immune response upon exposure [128]. Endotoxin is a component of Gram-negative bacterial cell walls and can contaminate nanomaterials during the manufacturing process or in handling. It has been shown that endotoxin can exacerbate inflammatory responses to nanoparticles [129-132]. As a result of the potent proinflammatory activity the presence of endotoxin in nanomedicines whose administration to individuals in an already diseased state leads to the question of how this, in combination with potential nanoparticle associated immunomodulation, may affect an already compromised immune system.

The formulation of nanomedicines can represent complicated, multistep processes often involving the use of volatile chemicals and reagents. These volatile agents must be removed to prevent toxicity being generated by carry-over from contaminants within the formulation process [133]. The cytotoxic analysis of a preparation of gold nanorods both pre- and post-purification has demonstrated the stark contrast which can be the result of residual manufacturing components [134]. This observation has also been described by some sources where the toxicological potential of carbon nanotubes has been assessed [135, 136]. The production of carbon nanotubes requires catalysis by transition metals [137]. Most frequently these are iron, nickel, and copper. As free ions, these metals have been shown to induce oxidative stress via the production of reactive oxygen species (**Figure 1**) [138, 139]. Chemical contamination of this type has been detected in commercially available preparations of carbon nanotubes where, following purification, the material was no longer deemed toxic [140].

Nanoparticle interference with assays

A number of *in vitro* assays have been adopted for use with nanomaterials [141]. Their translation to use in nanotoxicology is mainly due to their track record of versatility, simplicity, and reproducibility. As has become apparent in recent years; the appropriateness to apply these methodologies with little consideration to how novel materials may lead to spurious assay outcomes [142]. Determining the appropriateness of assays for this end is complicated by the intrinsic complexity of nanoparticles. As such, suitable inhibition/enhancement controls (IEC) should be included in this analysis when possible.

Adsorption of protein to the surface of nanoparticles reduces the concentration of free protein available for quantification. The polarity of nanoparticles can enhance or reduce their potential for binding proteins from a matrix. This is particularly evident by the reduction in measurable IL-8 due to adsorption to a titanium dioxide preparation [142]. Similarly, TLR9 and IL-1β binding to citrate-

stabilized gold nanoparticles has been documented [64, 65]. The ability of nanoparticles to interact with, and inactivate enzymes is a consideration which reaches beyond the potential *in vitro* and *in vivo* effects. Numerous methods for testing the toxicity of nanomaterials rely on enzymatic function. The potential for interaction dictates that further considerations be made so as not to generate data which may not be representative of the material but merely an artefact of experimental interference [143]. Few assays have been implicated with this form of interference to date. One that has been brought to light is the LDH assay. Inactivation of lactate dehydrogenase as a result of adsorption to nanoparticle surfaces has been presented as a mechanism by which the LDH assay can produce results which are not an accurate representation of nanoparticle action [142, 143].

Studying the haemotoxic effects of nanomaterials lends the opportunity for a number of methodological issues relating to the basic properties of nanoparticles under investigation. The turbidity of nanoparticle preparations is known to interfere with platelet aggregometry, the principal of which relies on the optical assessment of the decrease in turbidity due to platelet aggregation. A potential solution for this is to utilize alternative measurement methods such as flow cytometry. Systems utilising magnets, such as those used for measuring platelet activation, have the potential to be incompatible with magnetic nanoparticles. When subjected to the magnetic field a region of higher concentration may establish, the effect of which may skew any observations and not be representative of a uniform distribution.

Proliferation is commonly evaluated using the MTT assay, but there are numerous mechanisms by which this can be incompatible with nanomaterials. A potential issue with the use of this assay is that it relies on the metabolic conversion of the MTT compound. Materials which promote/alter mitochondrial biogenesis cause artificially high signal which could be mistaken as pro-proliferative [144]. Differences in rates of tetrazolium production is reflective of the metabolic state of the cells [145, 146]. It is known that activated lymphocytes are more metabolically active than non-activated, which may reflect altered metabolism rather than proliferation [147]. Nanoparticles affecting metabolism and proliferation would be difficult to discern so the use of further methods such as [3H]thymidine incorporation and CFSE should be utilised. Quantification of cytokines as a marker of proliferation can also be problematic as the reduction may be the result of cell death.

The issues described here hold equal validity not only for toxicity assays but for immunotoxicity as the reagents employ similar strategies for generation of a measurable result i.e. absorbance, fluorescence. As such, the potential for nanoparticle-based assay interference must be considered throughout assay development and data interpretation.

Nanoparticle physicochemical characteristics in biological matrices

In order to determine structure-activity relationships and define meaningful trends, it is necessary to accurately measure physicochemical characteristics. The application of nanomaterials under biological conditions, both *in vitro* and *in vivo*, require in-depth knowledge of their physicochemical properties in relevant matrices. Due to the increasing complexity of biological matrices, it is not sufficient to assume that characteristics determined under minimal conditions (i.e. under vacuum, or in water) are still valid in the rational design and development for given purposes. The size, charge, surface chemistry, stability, and a host of other properties can be directly and dramatically altered by the medium in which the nanoparticles are suspended, all of which may affect how the materials interact with biological processes [148, 149].

Not only is it important to produce accurate and appropriate determinations of the physicochemical characteristics of nanomaterials, but it must be appreciated that the production of such materials is often a complex multistep process. Changes in particle size and/or charge can affect particle biodistribution, immunological impact and broader aspects of safety for nanoparticles made of the same material [93, 100]. While polydispersity within and between preparations must be expected, this batch-to-batch variability must be strictly monitored and accounted for to minimize downstream issues.

The issue of determining biologically meaningful *in vitro* assays which can inform downstream *in vivo* studies is further complicated by the choice of appropriate cellular models and endpoints. A recent review by Dobrovolskaia [150] has examined these considerations in detail, as such will not be repeated here. Linked with this are need to choose relevant and efficacious controls as well as determine any interaction between the nanomaterial and assay itself. To exemplify this issue, it was earlier mentioned that numerous cytotoxicity assays are prone to nanoparticle-related interference. Without detailing the choice of cell line or endpoint the choice of controls and assay interaction potential shall be discussed. The cytotoxic compound of choice must be sufficiently potent within the given cell line to generate toxicity but would ideally have a mode of action similar to that which would be expected from a nanomaterial. While this is desirable, tetrazolium salts such as MTS/MTT which detect the REDOX potential of cells would not be necessarily compatible with ROS generators such as dicumarol which can lead to overestimation of cellular viability and proliferation [151]. Similarly, compounds which affect cell membrane integrity should be used with care in the LDH assay, especially when comparing results of different cytotoxicity assays. Cell-free preparations of assays can be considered vital as a means to not only generate a baseline but also to observe any concentration dependent interactions that may occur. This can be invaluable in fluorogenic assays such as DCF where a threshold for interference may exist [152]. As mentioned earlier, the inclusion of inhibition/enhancement controls can assist in determining whether observations are a result of cellular interactions with nanomaterials or solely due to the presence of the nanomaterial. This is becoming routine in limulus amoebocyte lysate (LAL)-based assays for measuring endotoxin in which a nanomaterial sample is spiked with a known amount of endotoxin and assessed for enhanced or diminished recovery [153]. The underlying principal is translatable to a host of assays in which inducers or inhibitors of the desired effect can be introduced in addition to nanomaterials. Although logical, these considerations are widely overlooked potentially resulting in misleading conclusions being drawn.

Considerations for specific patient populations

Research efforts examining the biocompatibility of nanomaterials primarily use blood, as well as immune cells, from healthy volunteers to assess potential interactions. However, the intended populations often have differential immunological profiles compared to healthy volunteers. It is, therefore, vital that these aspects be considered when testing novel engineered nanomaterials.

The broad concepts of immunological frailty and how they relate to potential interactions with nanomaterials has been described [154] and highlights the relative lack of experimental evidence in such populations compared to investigations in healthy volunteer cells and tissues. There is evidence to suggest that the genetic background of the test organism can influence the outcome of

457 biocompatibility testing. Gustafsson *et al.* [155], showed that the response to titanium dioxide
458 nanoparticles in rats was strain-specific, indicating that genetics plays a role in the response to
459 nanomaterials. Existing data on the effects of nanoparticles in animal models reflecting
460 immunological frailty, dysregulated immunity and immune-compromised states show that
461 nanoparticles can have greater, or an additive, toxicological effect to that resulting from the
462 diseased state [156]. However, how closely animal models can reflect the situation in humans with
463 respect to disease states is an ongoing issue surrounding many fields of research, and it seems likely
464 that obtaining *ex vivo* samples from patients with specific conditions may complement other pre-
465 clinical evaluations, prior to phase I trials.

466 As one would expect, potential side effects and immune interactions by nanomaterials may be
467 further influenced by dysregulation of the immune system as a result of the diseased state. HIV is a
468 pertinent example of this, wherein the disease is underpinned by complex multifactorial
469 immunomodulation, and treatment paradigms are currently being investigated for improvement via
470 the application of nanoformulation [157].

471 There exist several parallels between the immunological effects of nanomaterials and those of the
472 diseased state. These effects include some generated by chronic inflammation such as rheumatoid
473 arthritis, cancer, and even hepatitis and HIV.

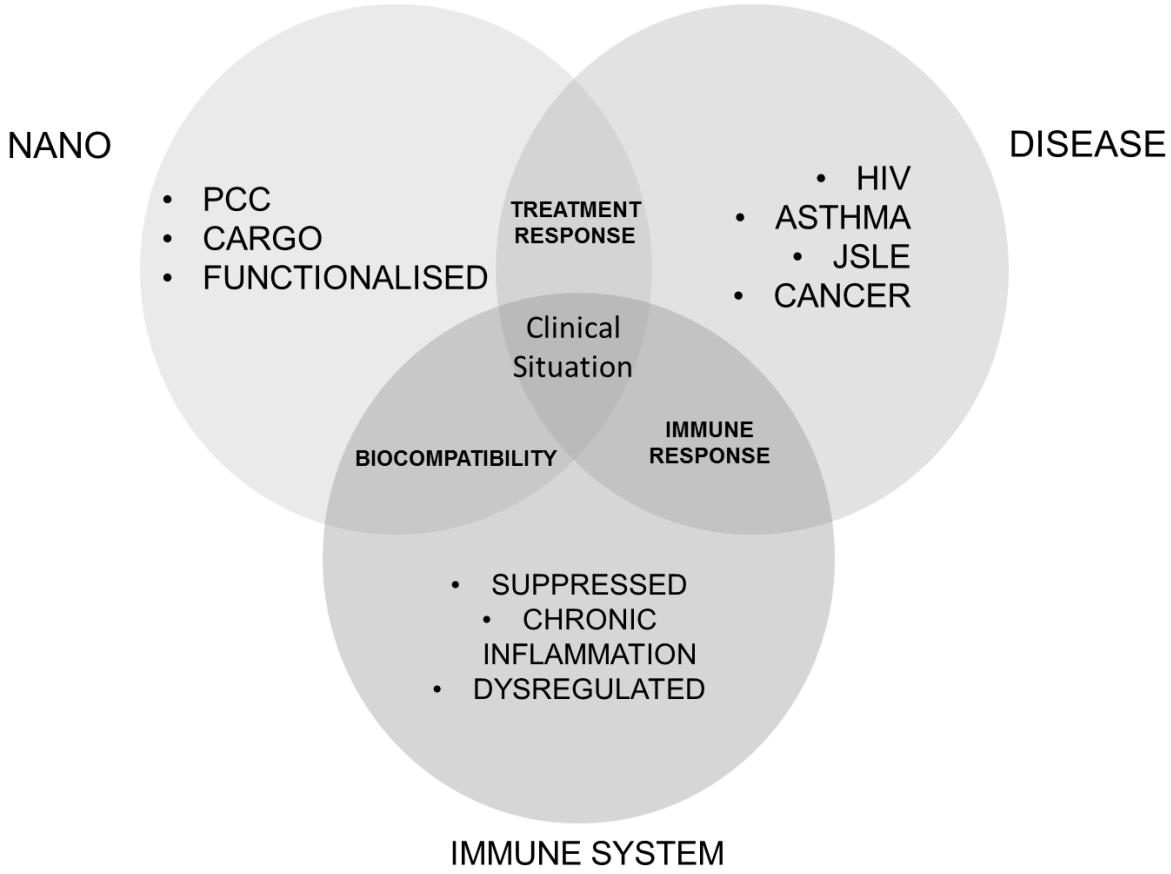
474 As mentioned previously, the activation of TH17 type response by TMC-TPP nanoparticles leads to
475 the generation of IL-17 [58]. The generation of this particular proinflammatory factor is of interest in
476 the pathogenesis of rheumatoid arthritis, as its production in the synovial tissue has been shown to
477 promote destructive collagen arthritis in an IL-1 independent manner in murine models [158], and
478 act synergistically with IL-1 and TNFα [159] .

479 The pathogenesis of cancer is intrinsically linked to a multitude of cytokines generated by the innate
480 and adaptive immune systems including IL-1, IL-6, IL-12, IFNγ, TNFα [160] all of which have been
481 shown to be associated with the interactions of various nanoparticles including silver (IL-1) [161],
482 MWCNT (IL-6) [162], and zinc oxide (IL-12, IFNγ, TNFα) [69]. As a platform for immunotherapy
483 nanoparticles are being studied due to their known induction of various immunostimulatory
484 cytokines which are proposed to exacerbate, and illicit, a greater immune response against
485 cancerous cells.

486 Mechanisms proposed to result in apoptosis in HCV and HIV-infected cells include loss of cell
487 membrane integrity, mitochondrial dysfunction and generation of ROS [163]. Silica [164] and
488 titanium dioxide [165] nanoparticles have been shown to alter cell membrane integrity in a charge-
489 and concentration-dependent manner. Oxidative stress and the generation of reactive oxygen
490 species is directly relatable to mitochondrial dysfunction (**Figure 1**) [166]. A large number of
491 nanomaterials have implicated with having a similar effect [167]. HIV has been shown to interfere
492 with the autophagic process via inhibition in dendritic cells, and induction in macrophages [168],
493 while HCV has shown to increase levels of autophagy in infected cells [169]. Inhibition [170] or
494 induction [171] of autophagy by nanomaterials (**Figure 1**) is also a commonality to the actions of HIV
495 and HCV. Therefore, it seems likely that certain material compositions should not be progressed for
496 certain applications.

Immunocompromised individuals can be defined as having a substantially weakened immune system, and this was originally thought to be the case in HIV infection. However, it is now known that the situation is not clear cut since a patients' immunological profile varies with the type of viral populations infecting them and their response to antiretroviral therapy [172]. Infection with HIV leads to a decline in CD4+ T cells, but treatment with antiretrovirals may produce resurgence in the number of these cells. However, it has been shown that although the number of CD4 T cells increases their functional capacity is diminished in chronic infection. This has been demonstrated by the increased expression of the receptor programmed death 1 (PD-1), a negative regulator of activated T-cells [173]. Cells expressing high levels of PD-1 were shown to be functionally exhausted compared to uninfected cells suggesting HIV+ patients are immunocompromised [174]. However, the reasons for this exhaustion of the immune system are unclear, and several hypotheses have been proposed [175]. An interesting hypothesis for the ongoing inflammation seen in HIV, which may be linked to T cell exhaustion, is the discovery that HIV itself can induce an inflammatory form of programmed cell death termed pyroptosis. Dotish *et al.* showed that HIV can directly induce pyroptosis in CD4 T cells via inflammasome activation and that this process could be blocked by inhibiting caspase-1 [176]. Interestingly, nanoparticles have been shown to interact with inflammasomes, NLRP3 in particular (**Figure 1**) [177] and carbon nanoparticles have been shown to induce pyroptosis [178]. This is an important consideration for the application of nanoparticles either in the treatment of HIV infection or when nanoparticles may be applied in HIV+ patients for concomitant health issues, e.g. raised cholesterol or infections. As a condition where chronic dosing is a reality which cannot be overlooked, the long term effects of any nanoformulation must be considered and is something we are investigating with interest.

Effects such as these may be tolerable in a healthy model but be potentially incompatible with the diseased state. It is also possible for the opposite to be true, where the observable effect is unacceptable under healthy conditions, whereas its effect on the diseased state may not be as pronounced and within a range where the potential benefits outweigh the negative outcomes. As is demonstrated in **Figure 2** the primary considerations of the nanomaterial itself, the immune system to which it will be introduced, and the disease on which it will act are not mutually exclusive. The intersections of biocompatibility and treatment response are those which weigh heavily in the development of nanomedicines. Often overlooked is the immune response relating the disease to the immune state, and also how the nanomaterial has influence over these. To be able to create a truly appropriate model for the design of nanomedicines, a holistic approach such as this must be adopted.



533

534 **Figure 2 - Key challenges in compatibility of nanoparticles as nanomedicines.** *Considerations*
535 *involved in the design, analysis, and application of nanomaterial for the treatment of disease linking*
536 *the material specific, immune state, and particular disease. A more holistic approach incorporating*
537 *investigation of immunological status and genetic variability in genes encoding immune signalling*
538 *proteins will allow a more holistic approach to the biocompatibility testing of novel engineered*
539 *nanomaterials. Acronyms used; PCC – physicochemical characteristics, HIV – human*
540 *immunodeficiency virus, JSLE – juvenile systemic lupus erythematosus.*

541

542 **US and EU efforts to promote the harmonization of nanoparticle testing**

543 To truly determine relationships between nanoparticle characteristics, the necessity to apply a more
544 standardised approach to assays has become apparent in order to correctly assess how
545 nanoparticles interact with biological systems. Many researchers involved in the development of
546 nanomaterials use well-defined assays to assess biocompatibility e.g. investigation of cytotoxicity by
547 using MTT assays. However, there are reports of contradictory test results from cell-based assays
548 [179, 180]. Unexpected variability can arise in such assays by differences in media composition,
549 passage time of cell lines and the source of the serum used in routine cell culture media. The
550 National Cancer Institute's Nanotechnology Characterisation Laboratory (NCI-NCL)
551 (http://ncl.cancer.gov/) has been at the forefront of promoting harmonisation of assays to
552 determine nanoparticle interactions with biological systems and offers standardised methodologies
553 for its assessment. Given the increasing development of nanomaterials across Europe, a need has
554 been identified to begin to regulate the preclinical evaluation of novel engineered nanomaterials as

well as provide a platform for the translation of these materials into clinical studies. The recently established European Nanomedicine Characterization Laboratory (EU-NCL) (http://www.euncl.eu/) shares the same ethos as the NCI-NCL in the provision of a standardised characterisation of nanomedicines to aid in their translation to the clinic and facilitate nanomedicine development. Currently, researchers and developers in Europe have to gather preclinical data from a multitude of non-integrated providers which may result in interlaboratory variability and, therefore, conflicting results. A major ambition of the EU-NCL is to tackle that obstacle by providing an open-access EU-wide characterisation infrastructure and maintain Europe as internationally competitive in nanomedicine development. EU-NCL offers a unique integrated solution ensuring access to high-quality data, experience, and facilities throughout Europe for a large range of medical applications. EU-NCL is a multi-centre infrastructure which is intended to overcome current fragmentation and to improve quality and efficiency of translation by drawing on expertise across Europe. The involvement of multiple analytical centres guarantees direct access to different domains in the nanomedicine communities and other stakeholders while maintaining the bandwidth to engage with Europe's most promising candidates. It is envisaged that using this integrated approach, EU-NCL will also be able to determine critical nanoparticle characteristics that relate to biological effects, without compromising confidentiality with developers. As such, this will enable researchers to access anonymised information to inform future rational design of nanomaterials.

Conclusions and future perspectives

The development, and implementation, of nanomaterials for a variety of clinical applications is increasing as their utility in improving healthcare is demonstrated. However, consideration must be given to appropriate pre-clinical testing to fully translate these materials into clinical use.

Numerous conclusions can be drawn from existing research, among which are perspectives on how pre-clinical testing can be improved from its current state. As mentioned here thorough physicochemical characterisation in biologically relevant matrices is vital, similarly assessing the contamination state of products. These need to be supported by biologically meaningful *in vitro* assays which can inform further *in vivo* studies. Linked with this are need to choose relevant and efficacious controls as well as determine any interaction between the nanomaterial and assay itself. Greater insight into the effect of nanoparticles on the diseased state would benefit from testing in relevant patient samples. Finally, the nanomaterials should be considered in the final format for which they have been developed. Not only will this aid in determining if the nanoparticle is fit for purpose, but also how its application may affect patient populations in terms of nanomedicine.

It is hoped that with greater integration and cooperation of various research efforts the development of nanomedicines will gain speed to bring forward these advances in patient care.

Executive Summary

Introduction

- Nanoformulation provides a platform which allows improvement over existing therapeutic and diagnostic tools.
- Physicochemical characteristics of nanomaterials can be tuned during the manufacturing process as a means to enhance/reduce physiological effects.
- Challenges in the characterisation of nanoparticles relating to biocompatibility relate to many factors including different manufacturing processes, and the immune state of the end user.

Interaction of nanoparticles with components of the immune system

- Various mechanisms, the biological purposes of which under normal circumstances are homeostatic or relating to clearance of pathogens, are known to be implicated following the introduction of nanomaterials to biological systems.
- While mechanisms of internalisation of nanomaterials differ as a result of cell type, as well as physiochemical characteristics i.e. size and charge, downstream effects such as the generation of reactive oxygen species etc. can be ubiquitous.
- Factors such as protein corona formation, although not well understood, are shown to modulate biological interactions, uptake, and overall pathophysiology.
- Inflammatory stimulation of the immune system, antibody production against certain materials are known examples of interactions which may be detrimental to the host.
- Immunosuppressive properties of certain nanomaterials associated with certain nanomaterials could potentially exacerbate the pathophysiology of immunocompromised individuals.
- Complexity in these considerations is increased by the presence of active pharmaceutical ingredients.

Interaction of nanoparticles with components of the blood

- Interactions of nanoparticles with haematological components can lead to modulation of thrombogenic potential.
- The complexity of these interactions is a function of the physicochemical characteristics of the nanomaterial as well as the multifactorial nature of the process of thrombogenesis.

Links between immunological and haematological systems

- The cooperation of immunological and haematological systems add complexity to the evaluation of nanomaterial biocompatibility.
- Leukocyte procoagulant activity is shown as an example where contamination of nanomaterial preparations can strongly generate a false positive.
- Complement and platelet activation are complex cascades both of which have been shown to be affected by the presence of various nanoparticles.
- Disruption of membrane integrity leading to haemolysis has been associated with a number of nanomaterials. The presence of a protein corona modulates this activity.

Challenges in assessing the biocompatibility of novel, engineered, nanoparticles

- The contamination state of tested materials, both biological and chemical, can skew data by the generation of false positives.
- The lack of nanoparticle-tailored assays necessitates the use of standard immunological assays, many of which succumb to interference by intrinsic properties of nanomaterials which can lead to spurious results.
- The necessity to utilize complementary assessment methodologies which focus on particular aspects via differential means has been highlighted.
- Physicochemical characterisation in biologically relevant matrices has been highlighted as providing a more relevant representation of the material coming in contact with cells.
- Suggestions have been provided relating to assay combinations and positive control choices.

Considerations for specific patient populations

- The immunological state of the intended recipient is of primary importance when considering the application of nanoparticles for nanomedicine.
- The immunological effects of nanoparticles have the potential to exacerbate those generated by the diseased state.
- Hallmarks of chronic inflammatory conditions display commonality with those generated by nanomaterials. As such caution must be taken in their use under such conditions.
- Assessment of nanomaterial safety is normally performed in healthy models and while convenient, does not provide the necessary conditions present in the diseased state.

US and EU efforts to promote the harmonization of nanoparticle testing

- International standardisation efforts for nanoparticle characterisation which can aid preclinical evaluation of nanomedicines by addressing the aforementioned challenges in nanomaterial testing

Conclusions and future perspectives

- The need for thorough and biologically relevant preclinical testing is reiterated.
- Consideration of the diseased state in these assessments is of high importance.

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